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Purification and characterization of a new alkaline serine protease from the thermophilic fungus *Myceliophthora* sp.

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ABSTRACT

This work reports the purification of a novel alkaline protease enzyme from a putative new thermophilic fungus *Myceliophthora* sp. The molecular weight of the enzyme was determined as 28.2 kDa by using MALDI-TOF MS and it was inhibited by PMSF indicating it is a serine-protease. The optimum pH and temperature were 9.0 and 40–45 °C, respectively. Mg was the only tested cation able to promote an increase of the protease activity. The N-terminal sequence of the purified protease (GVVGVC) presented identity and homology when compared to other proteases from fungi. This study also provides biochemical information about substrate specificity using fluorescence resonance energy transfer (FRET) peptide families derived from Abz-KLRSSKQ-EDDnp. The results showed that Abz-KLISSKQ-EDDnp is the best substrate among those tested for the purified protease (k_{cat}/K_m = 1275.3 mM^{-1} s^{-1}). Also, the specificity data suggest that subsites S_1, S_2, S_3 and S_1', S_2', S_3', in general, present a preference for hydrophobic residues with the exception of Glu in P_3, His in P_2' and Arg in P_3'. The highest values for the specificity constant k_{cat}/K_m were obtained for P_1, P_2 and P_2'.

1. Introduction

Proteases are the most important industrial enzymes, accounting for approximately 60% of the total industrial enzyme market [1]. The relevance of this group of enzymes, rich in structural diversity and mechanisms of action is reflected in the importance of their applications in industrial processes. They have multiple applications, for example in detergent formulations, textile, food, and pharmaceutical industries [2]. Therefore, the industrial demand of proteolytic enzymes, with appropriate specificity and stability to pH, temperature and chemical agents, continues to motivate the search for new sources.

Many microorganisms secrete proteases to the external environment in order to degrade proteins; their hydrolysis products are used as carbon and nitrogen sources for cell growth [3]. Proteases with elevated activity and stability in the high alkaline range are attractive for bioengineering and biotechnological applications, especially those from bacteria and fungi [4,5]. Alkaline proteases are used in the detergent industry because the pH of cleaning products is usually in the range of 9.0–12.0. Adding these enzymes to cleaning solutions allows the use of fewer toxic chemicals such as solvents and corrosive substances, decreasing their environmental impact [6].

Currently, the majority of proteases used in detergents are serine proteases [7] and their catalytic activity depends on the interplay of a nucleophile, a general base and an acid. In the two largest groups of serine-proteases, the (chymo)trypsin and subtilisin families, the catalytic triad is composed of serine, histidine and aspartate residues which exhibits similar spatial arrangements, but the order of the residues in the amino acid sequence and tertiary structure is different [8,9]. The active site performs the dual role of binding a substrate and catalyzing a reaction, and thus determines the specificity of the enzyme. Accordingly, it is possible to obtain information about the active site by analyzing the enzyme kinetics with different substrates and inhibitors [10].

Recently, we isolated a thermofilic fungus *Myceliophthora* sp. strain, which produces an extracellular proteolytic enzyme [11]. The present work reports the purification, biochemical and biophysical characteristics of the extracellular protease produced by *Myceliophthora* sp. using FRET peptides derived from sequence Abz-KLRSFKQ-EDDnp of the extracellular serine-protease produced by *Myceliophthora* sp. Also, we

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investigated the specificity of the subsites S_1, S_2, S_3 and S_1', S_2' and S_3' using FRET peptides derived from the sequence Abz-KLRSSKQ-EDDnp (Abz = o-aminobenzoic acid; EDDnp = N-[2,4 dinitrophenyl]ethylenediamine; Abz/EDDnp = donor/acceptor fluorescent pair).

2. Experimental procedures

2.1. Protease production in solid-state fermentation (SSF)

Erlenmeyer flasks (250 mL) containing media composed by 4.75 g of wheat bran and 0.25 g of casein and hydrated with 7 mL of distilled water and 3 mL of nutrient solution 0.1% (w/v) $(NH_4)_2SO_4$, $MgSO_4{\cdot}7H_2O$ and NH_4NO_3 were inoculated with 2 mL of a spore suspension and cultivated at 45 °C for 72 h. The fermented material was mixed with 30 mL of distilled water per 5 g of fermented material, stirred for 30 min, filtered and centrifuged at 10,000 × *g*, at 6 °C. The supernatant was used as a crude enzyme solution.

2.2. Protease activity

Proteolytic activity was assayed as described by Sarath et al. [12] with modifications. The reaction mixture was made up of 0.2 ml crude enzyme and 0.8 mL of 1% (w/v) casein dissolved in glycine buffer (50 mM, pH 9.0). The reaction was carried out at 50 °C and stopped after 30 min with 0.5 mL of 15% trichloroacetic acid (TCA). Test tubes were centrifuged at 15,000 × *g* for 30 min and the absorbance of the supernatant was measured at 280 nm using a Cary 100 (Varian) spectrophotometer. A control was prepared by adding TCA before the addition of the enzyme solution. One unit of enzyme activity (U mL^{-1}) was arbitrarily defined as the amount of enzyme required to cause an absorbance increase of 0.01 per minute at 280 nm under the assay conditions [13].

2.3. Purification protocol

The crude enzyme solution was concentrated using a precipitation procedure at 4 °C with ethanol (proportion 1:2). The crude precipitate was collected by centrifugation at 9000 × *g* for 30 min at 4 °C and then dissolved in 20 mM Tris pH 8.0 buffer with 0.2 M NaCl. The concentrated crude enzyme solution was then further purified by gel filtration on a Sephacryl S-100 column (1.6 cm × 75 cm) equilibrated with 20 mM Tris pH 8.0 containing 0.2 M NaCl. Fractions of 3 mL each were collected at a flow rate of 0.15 mL/min and analyzed for protease activity and protein concentration. Fractions exhibiting protease activities were pooled and applied to a Source 15-Q column (1 cm × 6 cm) that was previously equilibrated with 20 mM Tris, pH 8.5 buffer. Unbound proteins were removed with the equilibration buffer until the absorbance at 280 nm reached the baseline. Bound proteins were eluted with a linear gradient of sodium chloride in the range of 0–2.0 M in the equilibration buffer. Fractions of 1 mL each were collected at a flow rate of 1 mL/min and analyzed for protease activity and protein concentration.

2.4. Electrophoresis

Polyacrylamide gel electrophoresis under denaturing conditions was carried out to determine the purity and to estimate the molecular weight of the purified enzyme [14]. Protein bands were visualized after staining with silver nitrate. For the biochemical analysis described below we used the purified protease.

2.5. Mass spectrometry analysis

Protease molecular mass determination was carried out on a TOF Spec E mass spectrometer (Micromass, Manchester, UK) operating in linear mode using the matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) method, using α-cyano-4-hydroxycinnamic acid as the matrix [15].

2.6. Determination of protein concentration

Protein concentration was determined by the Bradford method [16], using bovine serum albumin as a standard.

2.7. FRET peptide synthesis

All the FRET peptides used for subsite mapping were synthesized by solid-phase synthesis and purified as described previously [17,18]. The molecular mass and purity of the peptides were confirmed by amino acid analysis and MALDI-TOF using a Microflex-LT mass spectrometer (Bruker—Daltonics, Billerica, MA, USA). Stock solutions of peptides were prepared in DMSO, and their concentrations were measured by colorimetric determination of the 2,4-dinitrophenyl group (molar extinction coefficient of 17.300 M^{-1} cm^{-1} at 365 nm) using a spectrophotometer Cary 100 (Varian) [19].

2.8. Kinetic measurements

The FRET peptides were assayed in a Shimadzu RF-5301 spectrofluorimeter at 45 °C in 50 mM glycine buffer, pH 9.0. The enzyme was pre-incubated in the assay buffer for 3 min before the addition of substrate. Fluorescence changes were monitored continuously at 320 nm excitation and 420 nm emission. The enzyme concentrations for initial rate determinations were chosen at a level intended to hydrolyze less than 5% of the amount of added substrate over the time course of data collection. The slope of the generated fluorescence signal was converted to micromoles of substrate hydrolyzed per minute based on a calibration curve obtained from the complete hydrolysis of each peptide. The kinetic parameters K_m and k_{cat} were calculated by nonlinear regression using the GraFit® software (Erithacus Software, Horley, Surrey, UK). Errors were less than 5% for each of the obtained kinetic parameters.

2.9. Effects of pH and temperature on enzyme activity

Optimum pH was determined by performing standard activity assays in a pH range from 3 to 10.5 at 45 °C using suitable buffers: sodium citrate dihydrate, sodium acetate, sodium phosphate, Hepes, Taps, glycine and Caps. In order to determine optimal temperature, the enzymatic assay was carried out at different temperatures (25–60 °C), at pH 9.0. The reactions were performed under pseudo first-order conditions ($[S] \ll K_m$) of hydrolysis of substrate Abz-KLRSFKQ-EDDnp. The data were fitted with the GraFit® software to the appropriate equation [20].

2.10. Effect of inhibitors on enzyme activity

The effects of inhibitors on protease activity were studied using PMSF, benzamidine, iodoacetic acid, EDTA and E-64. The enzyme was pre-incubated with inhibitors for 3 min at pH 9.0 and 45 °C, and then the remaining enzyme activity was estimated using Abz-KLRSFKQ-EDDnp as substrate. The reactions were performed under pseudo first-order conditions of hydrolysis of substrate. The activity of the enzyme assayed in the absence of inhibitors was considered as 100%.

2.11. Salt influence on enzyme activity

The influence of NaCl on the proteolytic activity was investigated up to 0.5 M. The protease was pre-incubated with NaCl for 3 min in pH 9.0, at 45 °C, and then the remaining enzyme activity was measured using Abz-KLRSFKQ-EDDnp as substrate. The reactions were performed under pseudo first-order conditions of hydrolysis of substrate. The activity of the enzyme assayed in the absence of salt was taken as 100%.

2.12. Effects of surfactants and oxidizing agents on enzyme activity

The effect of some surfactants (Triton X-100, Tween 20, Tween 80, and SDS) and oxidizing agents (DTT and β-mercaptoethanol) was studied by pre-incubating the protease with the agent for 3 min, pH 9.0, at 45 °C, prior to activity tests. The activity of the enzyme without any additive was taken as 100%. The reactions were performed under pseudo first-order conditions of hydrolysis of substrate.

2.13. Effect of divalent ions on enzyme activity

The protease was assayed in the presence of different metal ions including Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Hg^{2+}, and Ni^{2+} at 5 mM (final concentration). The reactions were performed under pseudo first-order conditions of hydrolysis of substrate. The activity of the enzyme assayed in the absence of divalent ions was taken as 100%.

2.14. Effect of organic solvents on enzyme activity

In order to determine the effect of organic solvents like methanol, ethanol, acetone, butanol and isopropanol (20%, v/v) on proteolytic activity the reactions were performed under pseudo first-order conditions of hydrolysis of substrate. The activity of the enzyme assayed in the absence of organic solvents was taken as 100%.

2.15. Determination of the substrate cleavage sites

The scissile bond of hydrolyzed peptides was identified by isolation of the fragments using analytical HPLC followed by determination of their molecular mass by LC/MS using an LCMS-2010 equipped with an ESI-probe (Shimadzu, Japan).

2.16. N-terminal sequence

The N-terminal sequence was determined by the Edman method [21] using the Protein Sequencer PPSQ-33A (Shimadzu Corporation, Kyoto, Japan). The PTH-amino acids were separated using HPLC, and they were identified and quantified by analyzing previously quantified standards and comparing retention times and UV absorption, respectively.

Table 1
Parameters of purification of a serine protease from thermophilic fungus *Myceliophthora* sp.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor (fold)	Recovery (%)
Crude Extract	2.49	19.80	8.0	1	100.0
Precipitation	1.25	15.75	12.6	1.6	79.5
Gel filtration	0.03	11.50	383.3	48.2	58.1
Ion exchange	0.01	10.2	1020.0	128.3	51.5

3. Results and discussion

3.1. Protease purification

The protease was purified by a three-step procedure which is summarized in Table 1. After ethanol precipitation, a Sephacryl S-100 HR column was used to purify the enzyme, obtaining four peaks (Fig. 1a). However, only the first peak (fractions 20–24) presented proteolytic activity. Only the fractions 23 and 24 were pooled and submitted to an anion exchange column Source 15-Q.

The anion exchange chromatography showed two peaks (Fig. 1b); the first one (A) eluted in the absence of NaCl containing unbound proteins and the second (B) eluted with a NaCl gradient of 0–2 M. The proteolytic activity was observed in peak A, indicating that the enzyme did not bind to the Source 15-Q and, therefore, would have a p*I* higher than 8.5 or maybe it is a glycosylated protein, a plausible reason for impaired binding. The enzyme was purified 128.3 fold with a final yield of 51.5% (Table 1), it was homogenous on SDS-PAGE, and its molecular weight was estimated to be 30.1 kDa (Fig. 1). Subsequently we performed a mass spectrometry (MALDI-TOF) to verify both purity and the molecular weight of the enzyme (results not shown). The data confirmed the purity of the protease and its exact molecular weight: 28.2 kDa (data not shown). The low molecular mass exhibited by the purified protease agrees with other works that report fungal proteases with a Mw lower than 50 kDa [22,23].

3.2. Effect of pH and temperature on enzyme activity

The effect of pH on the hydrolysis of Abz-KLRSFKQ-EDDnp is shown in Fig. 2a. The protease is more active in the pH range 7.0–10.5 with higher activity at pH 9.0 suggesting that it is an alkaline protease. Purified alkaline proteases from fungi have been described from *Clonostachys rosea* (pH 9) [24] and *Lecanicillium psalliotae* (pH 10) [25]. The temperature dependence was determined from 25 to 60 °C (Fig. 2b). Optimal hydrolysis occurred at 40–45 °C. Also, at 55 °C, the enzyme retained about 90% activity while at 60 °C, the activity reduced to 45%. Proteases from fungi show similar results for optimum temperature such as 45 °C for *Colletotrichum gloeosporioides* [26], 50 °C for *Trichoderma reesei* QM9414 [27] and *Fusarium culmorum* [28].

3.3. Effect of inhibitors on purified enzyme activity

The effect of some protease inhibitors was investigated on the hydrolysis of Abz-KLRSFKQ-EDDnp (Table 2). The assay was performed in pH 9.0 at 45 °C. Proteolytic activity was abolished by 5 mM PMSF (100% inhibition), suggesting that this enzyme would be a serine-protease. Other protease inhibitors like EDTA, benzamidine, iodoacetic acid and E-64 had none or weak effect on the activity. Serine proteases from thermofilic fungi have been reported by several authors such as Khan et al. [29] who reported purified serine protease from the thermophilic fungus *Paecilomyces lilacinus*, and by Charles et al. [30] and Hajji et al. [31], who reported purified proteases by thermotolerants fungi *Aspergillus nidulans* HA-10 and *Aspergillus clavatus* ES1, respectively.

Table 2
Effect of different types of inhibitors (5 mM) on the hydrolysis of Abz-KLRSFKQ-EDDnp by a serine protease from *Myceliophtora sp.*

Inhibitors	Relative activity (%)
Control	100
EDTA	95
PMSF	0
Benzamidine	100
E-64	80
Iodine-acetic acid	100

3.4. Salt influence on enzyme activity

The enzyme was slightly activated 8% in the presence of 250 mM NaCl, decreasing afterwards. Wang et al. [32] reported that 5 mM NaCl had minimal effect on the activity of a protease from *A. fumigatus*. Here we report that much higher concentrations of NaCl did not significantly enhance catalytic efficiency of the protease from *Myceliophthora*, consistent with Wang et al.'s findings. The lack of significant effects of sodium is drastically different from the behavior described for other serine proteases, especially for thrombin [33], a key enzyme from the blood coagulation cascade, where Na^+ exerts an allosteric role and drastically improves the catalytic properties towards its natural substrate fibrinogen.

3.5. Effect of surfactants and oxidizing agents on protease activity

The effect of surfactants and reducing agents on protease activity is shown in Table 3. The enzyme presented a small loss of activity in the presence of the non-ionic surfactant Triton X-100 and it was activated in the presence of Tween 20. However, in the presence of

Table 3
Effect of reducing agents and surfactants on the hydrolysis of Abz-KLRSFKQ-EDDnp by a serine protease from *Myceliophthora* sp.

	Final concentration (mM or v/v)	Relative activity (%)
Control	–	100.0
β-Mercaptoethanol	2 mM	91.5
DTT	2 mM	82.9
Tween 20	1%	117.2
Triton X-100	1%	88.6
Tween 80	1%	20.0
SDS	0.1%	0.0

Table 4
Effect of divalent ions (5 mM final concentration) on the hydrolysis of Abz-KLRSFKQ-EDDnp by a serine protease from *Myceliophthora* sp.

Compound	Relative activity (%)
Control	100.0
Mg^{2+}	122.2
Ca^{2+}	97.2
Ba^{2+}	94.4
Mn^{2+}	91.7
Zn^{2+}	77.8
Ni^{2+}	41.7
Hg^{2+}	33.3
Cu^{2+}	8.3

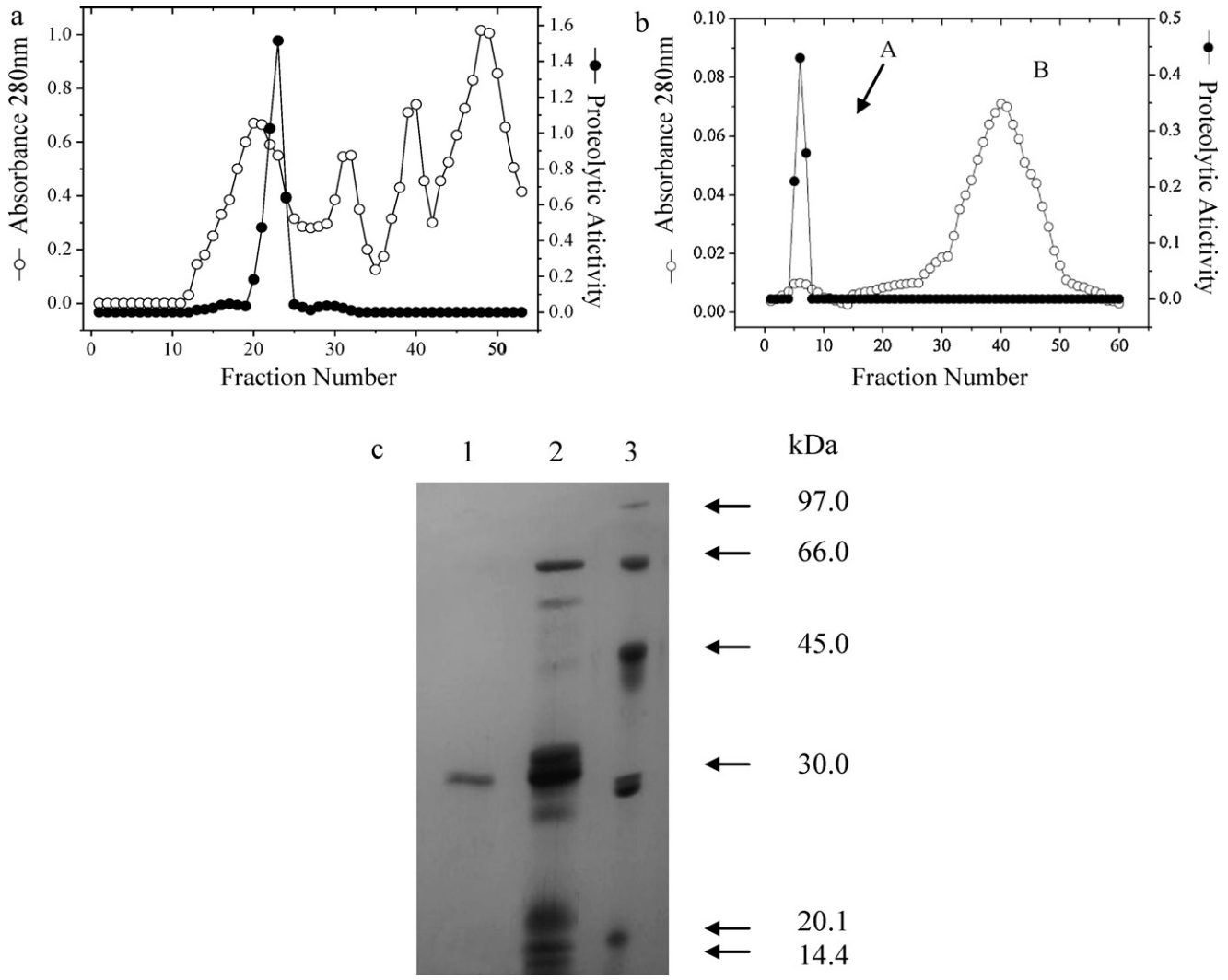

Fig. 1. Closed circles: proteolytic activity, open circles: protein profile at 280 nm (a) gel filtration chromatography on Sephacryl S-100 HR. Conditions: 20 mM Tris buffer, 0.2 M NaCl, pH 8.0; (b) protease ion exchange chromatography on Source 15-Q (fractions 23 and 24 gel filtration). Peak A: the protease elutes in the absence of the saline gradient. Peak B: elutes during the linear gradient from 0 to 1 M of NaCl. Conditions: 20 mM Tris buffer, pH 8.5. (c) SDS-PAGE 12%. Lane 1, pure protease; lane 2, crude extract and lane 3, molecular weight markers: α-lactalbumin (14.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), and phosphorylase b (97 kDa).

other non-ionic surfactants with a longer chain such as Tween 80, it displayed only 20% of proteolytic activity. Also, the protease was completely inactivated by the anionic surfactant SDS.

The activity of alkaline protease from *Myrothecium verrucaria* is only slightly affected by the presence of 2.5% Triton 100 (13%) [34]. The increase of activity by Tween 20, was also reported for the serine protease from *C. rosea* [23], which exhibited 16% of activation by Tween 20. Also, the serine protease from *A. clavatus* ES1 [31] presented about 33% of residual activity in the presence of 0.5% SDS, a higher concentration than the one we tested. On the

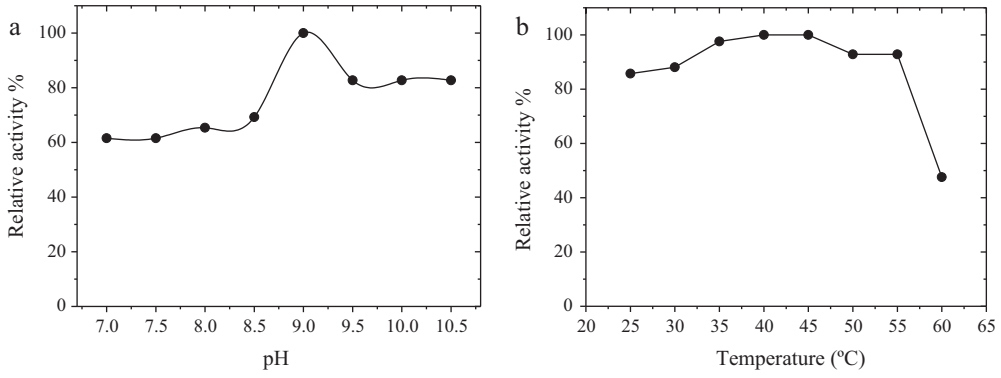

Fig. 2. The influence of (a) pH and (b) temperature on hydrolysis of Abz-KLRSFKQ-EDDnp by the purified protease.

Table 5
Effect of organic solvents (20%) on the hydrolysis of Abz-KLRSFKQ-EDDnp by a serine protease from *Myceliophthora* sp.

	Relative activity (%)
Control	100.0
Acetone	0.0
Butanol	0.0
Ethanol	47.2
Methanol	38.9
Isopropanol	91.7

other hand, thiol reducing agents such as DTT, which disassociate disulfide bonds (34) crucial for stabilizing the tertiary structure of proteins [35]. DTT and β-mercaptoethanol did not affect the protease activity significantly. Also, the trypsin like protease from *Cordyceps militaris* in the presence 1 mM DTT did not suffer significant activity loss [36]. In contrast, the activity of serine protease from *Cordyceps sinensis* had it activity increased in the presence 2 mM DTT and β-mercaptoethanol: 29.8% and 14.5%, respectively [37].

Table 6
Kinetic parameters for the hydrolysis of the FRET peptide series Abz-KLXSSKQ-EDDnp by a serine protease from *Myceliophthora* sp. for the characterization of its S_1, S_2, S_3 subsite specificity. The assays were performed at 45 °C in 50 mM glycine buffer, pH 9.0. The arrow indicates the cleaved peptide bond.

Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} mM^{-1})
Abz-KLX↓SSKQ-EDDnp (P_1)			
Ile	0.12	0.09	1275
Met	0.78	1.15	676
Trp	0.82	1.28	639
Ala	0.97	3.77	259
Phe	2.16	9.37	230
Thr	1.31	7.58	173
Lys	1.44	8.88	162
(Reference) Arg	0.61	4.60	133
KLPSSK↓Q Pro	0.01	0.08	121
Asn	0.41	3.88	105
Ser	0.99	10.63	93
Leu	0.38	6.80	56
Gln	0.16	3.10	53
His	0.17	4.82	35
Val	0.07	4.81	14
Cys	0.04	5.25	7
Gly		No hydrolysis	
Abz-KXR↓SSKQ-EDDnp (P_2)			
Phe	0.04	0.06	683
Pro	0.02	0.07	299
(Reference) Leu	0.61	4.60	133
Val	0.44	5.08	86
Ala	0.25	6.80	37
Ile	0.37	10.70	35
Tyr	0.05	1.96	25
Thr	0.17	7.19	24
Gln	0.10	5.50	18
Gly	0.03	2.30	12
Asp	0.07	8.46	12
Asn	0.02	2.42	7
His		No hydrolysis	
Abz-XLR↓SSKQ-EDDnp (P_3)			
(Reference) Lys	0.61	4.60	133
Asp	0.71	6.16	115
Ile	0.36	4.02	89
Phe	0.57	6.70	86
Val	0.25	4.67	55
Gln	0.14	5.87	24
Leu	0.20	8.52	24
Arg	0.13	5.80	23
Ala	0.07	3.34	22
Asn	0.06	3.39	18
His	0.08	4.90	16
Glu	0.07	4.67	16

Table 7
Kinetic parameters for the hydrolysis of the FRET peptide series Abz-KLRSSKQ-EDDnp by a serine protease from *Myceliophthora* sp. for the characterization of its S_1', S_2', S_3' subsite specificity. The assays were performed at 45 °C in 50 mM glycine buffer, pH 9.0. The arrow indicates the cleaved peptide bond.

Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} mM^{-1})
Abz-KLR↓XSKQ-EDDnp (P_1')			
(Reference) Ser	0.61	4.60	133
Phe	0.02	0.45	41
Ile	0.02	0.57	34
Gly	0.16	5.70	28
Gln	0.05	2.00	28
Glu	0.17	7.10	24
His	0.15	7.72	20
Arg	0.07	4.81	14
Pro		No hydrolysis	
Abz-KLR↓SXKQ-EDDnp (P_2')			
Phe	1.42	4.28	332
His	0.58	2.24	261
Leu	0.17	1.02	164
(Reference) Ser	0.61	4.60	133
Ala	0.77	7.30	106
Val	0.15	3.00	51
Gly	0.03	0.66	50
Asn	0.11	2.28	46
Arg	0.05	1.20	39
Pro		No hydrolysis	
Abz-KLR↓SSXQ-EDDnp (P_3')			
(Reference) Lys	0.61	4.60	133
Arg	0.04	0.80	52
Val	0.04	1.30	29
Gln	0.11	4.41	24

3.6. Effect of divalent ions on enzyme activity

Table 4 shows the effect of divalent ions. The addition of $MgCl_2$ increased enzyme activity by about 22% compared to the control. Ca^{2+}, Ba^{2+} and Mn^{2+} showed little influence on enzyme activity. Zn^{2+} exerted more influence decreasing activity by 22%, whereas Hg^{2+} and Ni^{2+} significantly inhibited the enzyme activity with pronounced inhibition. Also, with addition of Cu^{2+}, the purified protease was inhibited approximately by 92%. A serine-protease from *A. clavatus* ES1 was activated in the presence of 5 mM Mg^{2+} [31], and serine proteases from *Scedosporium apiospermum* [38] and *C. sinensis* [37] were also inhibited in the presence of 5 mM Cu^{2+}. This metal can react with cysteines promoting their oxidation and formation of cystine (Cys–Cys) [39], probably affecting enzyme structure and/or the access to the active site.

3.7. Effect of organic solvents on enzyme activity

In the presence of acetone and butanol, the enzyme activity was completely inhibited. Ethanol and methanol caused inhibition of more than 50% in the enzyme: 47.2% and 38.9% of baseline activity, respectively. By contrast, addition of isopropanol only reduced activity to 91.7% (Table 5). These results differ markedly from those of the serine protease from *Aspergillus fumigatus* [26] which presented 78% of its activity in the presence of 50% ethanol.

3.8. Determination of the substrate cleavage sites

FRET peptides derived from Abz-KLRSSKQ-EDDnp: The FRET peptide series Abz-**X**LRSSKQ-EDDnp, Abz-K**X**RSSKQEDDnp, Abz-KL**X**SSKQ-EDDnp, Abz-KLR**X**SKQ-EDDnp, Abz-KLRS**X**KQ-EDDnp and Abz-KLRSS**X**Q-EDDnp where X is the varying residue, were taken as a reference to explore the specificity of the subsites S_3, S_2, S_1, and S_1', S_2', S_3', respectively. Assays were performed in 50 mM glycine, pH 9.0 at 45 °C. The kinetic parameters k_{cat}, K_m and specificity constant (k_{cat}/K_m) for their hydrolysis by the protease

Table 8
Amino terminal sequence of protease as compared to other proteases using NCBI Blast Databank.

Protein	Sequence	NCBI Blast
Serine protease (*Myceliophthora* sp.)	**G V V G V C**	–
Serine protease (*Metarhizium flavoviride var. minus*)	S **V V G V** Q	gb|ACT66133.1|
Serine protease (*Metarhizium album*)	S **V V G V** Q	gb|ACT66132.1|
Serine protease (*Metarhizium anisopliae*)	S **V V G V** Q	gb|ACT66131.1|
Serine protease (*Metarhizium majus*)	S **V V G V** Q	gb|ACT66127.1|
Protease (*Coccidioides immitis RS*)	L **V V G V** I	gb|EAS33583.1|
Trypsin-like protease (*Septobasidium carestianum*)	I **V V G V** S	gb|AAR91723.1|
Protease (*Candida glabrata*)	S **V V G V** R	emb|CAG61409.1|
Serine protease (*Monacrosporium haptotylum*)	**G V V G** R R	gb|ABV46590.1|
Protease (*Verticillium dahliae*)	**G V V G** A S	gb|AAR10769.1|
Protease (*Methylocella silvestris BL2*)	**G V V G V C**	gb|ACK49719.1|
Serine protease (*Aedes aegypti*)	**G V** I **G V C**	gb|EAT46743.1|

are shown in Tables 6 and 7. The hydrolysis of FRET peptides of the six tested series showed a clear preference (analyzed in terms of the specificity constant k_{cat}/K_m) for hydrophobic residues at P_1, P_2 and P_2', which also displayed the highest values for k_{cat}/K_m.

Analyzing the protease behavior in relation to substrate specificity, Table 6 shows globally a preferential single cleavage occurred at the side of the carbonyl carbon of P_1. The only exception was Pro in P_1 position, that showed a displaced cleavage reaching the C side of P_3'. However, it is relevant to point out that for most substrates, the enzyme showed a second point of cleavage (accounting for 5–10% of the product concentration) at the C side of P_2' position (results not shown). For P_1, the protease displayed a greater preference for the hydrophobic residues Ile > Met > Trp, followed by Ala and Phe.

The catalytic specificity for Ile was almost tenfold higher than for the reference peptide, which has Arg at P_1. Compared to values reported by the literature, the serine protease from *Myceliophthora* sp. was unable to show a preferential cleavage at S_1 for trypsin-type substrates (Arg/Lys) and displayed significant activity only against Trp among the characteristic preferred residues Tyr/Phe/Trp/Leu [40]. An alkaline serine protease isolated from the photosynthetic bacterium *Rubrivivax gelatinosus* KDDS1 [41] exhibited a preference for Met at this position, but low activity for Trp and Ile. Thus, the preference of the alkaline serine protease from *Myceliophthora* sp. differs from the alkaline serine protease from *T. reesei* QM9414, classified as being a trypsin-like enzyme [27].

For P_2 (Table 6), the highest specificity constant was obtained for the hydrophobic residues Phe and Pro, followed by the reference peptide (Leu). For P_3 the highest preference was for the reference peptide (Lys) followed by Glu, Ile and Phe, showing poor selectivity. For P_1' (Table 7) the preference was for the reference peptide (Ser) followed by the hydrophobic residues Phe and Ile. For P_2' (Table 7), the preference was again for the hydrophobic residue Phe followed by the basic residue His; however, at pH 9.0 His predominates in the uncharged form. In P_3' (Table 7), only the reference peptide (Lys) displayed higher values of k_{cat}/K_m.

3.9. N-terminal sequence

The sequence of 6 residues at the N-terminal of the purified protease was determined and similar peptides retrieved by BLASTp searches of GenBank. Table 8 shows that the sequenced protease has identity and conserved residues with some proteases from others fungi. Also, the sequence of this protease has identity with the proteases from *Methylocella silvestris* BL2 (unpublished) and a serine protease from *Aedes aegypti* [42].

In conclusion, the protease purified from *Myceliophthora* sp. presents functional and stability properties that could be interesting for applications in food industries or detergent formulations, motivating us to perform tests for specific applications.

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